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(54) Title: SOLUBLE, CLEAVABLE SUBSTRATES OF THE HEPATITIS C VIRUS PROTEASE (57) Abstract Soluble HCV nonstructural substrates of the HCV polyprotein.		

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SOLUBLE, CLEAVABLE SUBSTRATES OF THE
HEPATITIS C VIRUS PROTEASE

BACKGROUND OF THE INVENTION

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Hepatitis C virus (HCV) is considered to be the major etiological agent of non-A non-B (NANB) hepatitis, chronic liver disease, and hepatocellular carcinoma (HCC) around the world. The viral infection accounts for greater than 90% of transfusion -associated hepatitis in U.S. and it is the predominant form of hepatitis in adults over 40 years of age. Almost all of the infections result in chronic hepatitis and nearly 20% develop liver cirrhosis.

The virus particle has not been identified due to the lack of an efficient *in vitro* replication system and the extremely low amount of HCV particles in infected liver tissues or blood. However, molecular cloning of the viral genome has been accomplished by isolating the messenger RNA (mRNA) from the serum of infected chimpanzees then cloned using recombinant methodologies. [Grakoui A. *et al.* J. Virol. 67: 1385 - 1395 (1993)] It is now known that HCV contains a positive strand RNA genome comprising approximately 9400 nucleotides, whose organization is similar to that of flaviviruses and pestiviruses. The genome of HCV, like that of flavi- and pestiviruses, encodes a single large polyprotein of about 3000 amino acids which undergoes proteolysis to form mature viral proteins in infected cells.

Cell-free translation of the viral polyprotein and cell culture expression studies have established that the HCV polyprotein is processed by cellular and viral proteases to produce the putative structural and nonstructural (NS) proteins. At least nine mature viral proteins are produced from the polyprotein by specific proteolysis. The order and nomenclature of the cleavage products are as follows: NH₂-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH.(Fig 1). The three

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amino terminal putative structural proteins, C (capsid), E1, and E2 (two envelope glycoproteins), are believed to be cleaved by host signal peptidases of the endoplasmic reticulum(ER) . The host enzyme is also responsible for generating the amino terminus of NS2 . The proteolytic processing of the nonstructural proteins are carried out by the viral proteases: NS2-3 and NS3, contained within the viral polyprotein. The NS2-3 protease catalyzes the cleavage between NS2 and NS3. It is a metalloprotease and requires both NS2 and the protease domain of NS3. The NS3 protease catalyzes the rest of the cleavages of the substrates in the nonstructural part of the polyprotein. The NS3 protein contains 631 amino acid residues and is comprised of two enzymatic domains: the protease domain contained within amino acid residues 1-181 and a helicase ATPase domain contained within the rest of the protein. It is not known if the 70 kD NS3 protein is cleaved further in infected cells to separate the protease domain from the helicase domain, however, no cleavage has been observed in cell culture expression studies.

The NS3 protease is a member of the serine class of enzymes. It contains His, Asp, and Ser as the catalytic triad, Ser being the active site residue. Mutation of the Ser residue abolishes the cleavages at substrates NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B. The cleavage between NS3 and NS4A is intramolecular, whereas the cleavages at NS4A/4B, 4B/5A, 5A/5B sites occur in *trans* .

Experiments using transient expression of various forms of HCV NS polyproteins in mammalian cells have established that the NS3 serine protease is necessary but not sufficient for efficient processing of all these cleavages. Like flaviviruses, the HCV NS3 protease also requires a cofactor to catalyze some of these cleavage reactions. In addition to the serine protease NS3, the NS4A protein is absolutely required for the cleavage of the substrate at the 4B/5A site and increases the efficiency of cleavage of the substrate between 5A/5B, and possibly 4A/4B.

Because the HCV NS3 protease cleaves the non-structural HCV proteins which are necessary for the HCV replication, the NS3 protease can be a target for the development of therapeutic agents against the HCV virus. The gene encoding the HCV NS3 protein has been cloned

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as disclosed in U.S. Patent No. 5,371,017, however, it has not been expressed in soluble, active form which is useful to discover inhibitors of the NS3 protease. Also the substrates 4A/4B, 4B/5A and 5A/5B have been cloned but not expressed in soluble active form useful to discover
5 inhibitors of the NS3 protease. If the HCV protease is to be useful as a target in a screen to discover therapeutic agents, both the protease and substrates must be in soluble active form. Thus, there is a need for a soluble active form of the HCV protease substrates which can be produced in large quantities to be used in a high throughput screen to
10 discover inhibitors of the protease and for structural studies.

SUMMARY OF THE INVENTION

The present invention fills this need by providing for soluble
15 HCV substrates which comprise the nonstructural polyprotein cleavage sites of HCV. The substrate peptides are made soluble by attaching a solubilizing motif to the peptide. In particular the sequences of the substrates defined by SEQ ID NOs: 16, 17, 18, 19, 20, and 21 are claimed.

BRIEF DESCRIPTION OF THE FIGURES

- 20 Figure 1 schematically depicts the HCV polyprotein.
- Figure 2 depicts the recombinant synthesis of plasmid pBJ1015.
- 25 Figure 3 depicts the recombinant synthesis of plasmid pTS56-9.
- Figure 4 depicts the recombinant synthesis of plasmid pJB1006.
- 30 Figure 5 depicts the recombinant synthesis of plasmid pBJ1022.
- Figure 6 depicts the recombinant synthesis of plasmid pNB(-V)182Δ4AHT.
- 35 Figure 7 depicts the recombinant synthesis of plasmid pT5His/HIV/183.
- Figure 8 schematically depicts a high throughput assay for discovering HCV protease inhibitors using surface plasmon resonance technology.

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DETAILED DESCRIPTION OF THE INVENTION

The teachings of all references cited are incorporated herein in their entirety by reference.

10 The present invention is soluble form of the HCV nonstructural polyproteins which are substrates for the HCV NS3 protease. The HCV NS3 protease cleaves the polyprotein and separates the 4A/4B, 4B/5A, and 5A/5B regions of the HCV polyprotein. One can use the noncleaved substrates to assay for protease inhibitors. Using the
15 scintillation proximity assay or the surface plasmon assay described below, one can determine whether or not the HCV protease has cleaved the substrate which is used. If the substrate is not cleaved, then the substance which is being tested is an HCV protease inhibitor. While on the other hand, if the substrate is cleaved then the substance which is
20 being tested is not a protease inhibitor. The substrates of the present invention are made soluble by attaching a solubilizing motif onto the substrate. Examples of solubilizing motifs are ionizable amino acids such as arginine and lysine.

25 The substrates 5A/5B and 4B/5A can be synthesized by a suitable method such as by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. The polypeptides are preferably prepared by solid phase peptide synthesis as described by Merrifield, J. Am. Chem. Soc. 85:2149 (1963). The synthesis
30 is carried out with amino acids that are protected at the alpha-amino terminus. Trifunctional amino acids with labile side-chains are also protected with suitable groups to prevent undesired chemical reactions from occurring during the assembly of the polypeptides. The alpha-amino protecting group is selectively removed to allow subsequent
35 reaction to take place at the amino-terminus. The conditions for the removal of the alpha-amino protecting group do not remove the side-chain protecting groups.

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The alpha-amino protecting groups are those known to be useful in the art of stepwise polypeptide synthesis. Included are acyl type protecting groups (*e.g.*, formyl, trifluoroacetyl, acetyl), aryl type protecting groups (*e.g.*, biotinyl), aromatic urethane type protecting groups [*e.g.*, benzyloxycarbonyl (Cbz), substituted benzyloxycarbonyl and 9-fluorenylmethyloxy-carbonyl (Fmoc)], aliphatic urethane protecting groups [*e.g.*, t-butyloxycarbonyl (tBoc), isopropylloxycarbonyl, cyclohexyloxycarbonyl] and alkyl type protecting groups (*e.g.*, benzyl, triphenylmethyl). The preferred protecting groups are tBoc and Fmoc, thus the peptides are said to be synthesized by tBoc and Fmoc chemistry, respectively.

The side-chain protecting groups selected must remain intact during coupling and not be removed during the deprotection of the amino-terminus protecting group or during coupling conditions. The side-chain protecting groups must also be removable upon the completion of synthesis, using reaction conditions that will not alter the finished polypeptide. In tBoc chemistry, the side-chain protecting groups for trifunctional amino acids are mostly benzyl based. In Fmoc chemistry, they are mostly tert.-butyl or trityl based.

In tBoc chemistry, the preferred side-chain protecting groups are tosyl for Arg, cyclohexyl for Asp, 4-methylbenzyl (and acetamidomethyl) for Cys, benzyl for Glu, Ser and Thr, benzyloxymethyl (and dinitrophenyl) for His, 2-Cl-benzyloxycarbonyl for Lys, formyl for Trp and 2-bromobenzyl for Tyr. In Fmoc chemistry, the preferred side-chain protecting groups are 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) or 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, trityl for Asn, Cys, Gln and His, tert-butyl for Asp, Glu, Ser, Thr and Tyr, tBoc for Lys and Trp.

For the synthesis of phosphopeptides, either direct or post-assembly incorporation of the phosphate group is used. In the direct incorporation strategy, the phosphate group on Ser, Thr or Tyr may be protected by methyl, benzyl or tert.butyl in Fmoc chemistry or by methyl, benzyl or phenyl in tBoc chemistry. Direct incorporation

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of phosphotyrosine without phosphate protection can also be used in Fmoc chemistry. In the post-assembly incorporation strategy, the unprotected hydroxyl group of Ser, Thr or Tyr was derivatized on solid phase with di-tert-butyl-, dibenzyl- or dimethyl-N,N'-diisopropylphosphoramidite and then oxidized by tert-butylhydroperoxide.

Solid phase synthesis is usually carried out from the carboxyl-terminus by coupling the alpha-amino protected (side-chain protected) amino acid to a suitable solid support. An ester linkage is formed when the attachment is made to a chloromethyl, chlorotriyl or hydroxymethyl resin, and the resulting polypeptide will have a free carboxyl group at the C-terminus. Alternatively, when an amide resin such as benzhydrylamine or p-methylbenzhydrylamine resin (for tBoc chemistry) and Rink amide or PAL resin (for Fmoc chemistry) is used, an amide bond is formed and the resulting polypeptide will have a carboxamide group at the C-terminus. These resins, whether polystyrene- or polyamide-based or polyethyleneglycol-grafted, with or without a handle or linker, with or without the first amino acid attached, are commercially available, and their preparations have been described by Stewart et al (1984), *"Solid Phase Peptide Synthesis"* (2nd Edition), Pierce Chemical Co., Rockford, IL.; and Bayer & Rapp (1986) *Chem. Pept. Prot.* 3, 3; and Atherton, et al. (1989) *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press, Oxford.

The C-terminal amino acid, protected at the side-chain if necessary and at the alpha-amino group, is attached to a hydroxymethyl resin using various activating agents including dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide (DIPCDI) and carbonyldiimidazole (CDI). It can be attached to chloromethyl or chlorotriyl resin directly in its cesium tetramethylammonium salt form or in the presence of triethylamine (TEA) or diisopropylethylamine (DIEA). First amino acid attachment to an amide resin is the same as amide bond formation during coupling reactions

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Following the attachment to the resin support, the alpha-amino protecting group is removed using various reagents depending on the protecting chemistry (e.g., tBoc, Fmoc). The extent of Fmoc removal can be monitored at 300-320 nm or by a conductivity cell. After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the required order to obtain the desired sequence.

Various activating agents can be used for the coupling reactions including DCC, DIPCDI, 2-chloro-1,3-dimethylimidium hexafluorophosphate (CIP), benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and its pyrrolidine analog (PyBOP), bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBroP), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and its tetrafluoroborate analog (TBTU) or its pyrrolidine analog (HBPYU), O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and its tetrafluoroborate analog (TATU) or pyrrolidine analog (HAPYU). The most common catalytic additives used in coupling reactions include 4-dimethylaminopyridine (DMAP), 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HODhbt), N-hydroxybenzotriazole (HOBt) and 1-hydroxy-7-azabenzotriazole (HOAt). Each protected amino acid is used in excess (>2.0 equivalents), and the couplings are usually carried out in N-methylpyrrolidone (NMP) or in DMF, CH₂Cl₂ or mixtures thereof. The extent of completion of the coupling reaction can be monitored at each stage, e.g., by the ninhydrin reaction as described by Kaiser *et al.*, *Anal. Biochem.* 34:595 (1970). In cases where incomplete coupling is found, the coupling reaction is extended and repeated and may have chaotropic salts added. The coupling reactions can be performed automatically with commercially available instruments such as ABI model 430A, 431A and 433A peptide synthesizers.

After the entire assembly of the desired peptide, the peptide-resin is cleaved with a reagent with proper scavengers. The Fmoc peptides are usually cleaved and deprotected by TFA with scavengers (e.g., H₂O, ethanedithiol, phenol and thioanisole). The tBoc peptides

are usually cleaved and deprotected with liquid HF for 1-2 hours at -5 to 0°C, which cleaves the polypeptide from the resin and removes most of the side-chain protecting groups. Scavengers such as anisole, dimethylsulfide and p-thiocresol are usually used with the liquid HF to prevent cations formed during the cleavage from alkylating and acylating the amino acid residues present in the polypeptide. The formyl group of Trp and dinitrophenyl group of His need to be removed, respectively, by piperidine and thiophenol in DMF prior to the HF cleavage. The acetamidomethyl group of Cys can be removed by mercury(II) acetate and alternatively by iodine, thallium (III) trifluoroacetate or silver tetrafluoroborate which simultaneously oxidize cysteine to cystine. Other strong acids used for tBoc peptide cleavage and deprotection include trifluoromethanesulfonic acid (TFMSA) and trimethylsilyltrifluoroacetate (TMSOTf).

Recombinant DNA methodology can also be used to prepare the polypeptide substrates. The known genetic code, tailored if desired with known preferred codons for more efficient expression in a given host organism, can be used to synthesize oligonucleotides encoding the desired amino acid sequences. The phosphoramidite solid support method of Matteucci *et al.*, *J. Am. Chem. Soc.* 103:3185 (1981) or other known methods can be used for such syntheses. The resulting oligonucleotides can be inserted into an appropriate vector and expressed in a compatible host organism.

The peptides of the invention can be purified using HPLC, gel filtration, ion exchange and partition chromatography, countercurrent distribution or other well known methods.

Also disclosed is the production of the HCV NS3 protease in a soluble form. The HCV NS3 protease must be in a soluble form to be used in a screen to detect compounds which inhibit the protease from cleaving its target substrate. We have discovered that if a peptide containing a solubilizing motif is attached to either the NS3 protease, preferably to the carboxyl terminus, the NS3 protease becomes readily soluble.

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The amino acid sequence of the NS3 protease catalytic domain is shown in SEQ ID NO: 1. Prior to the present invention the NS3 protease was not expressed in a cell in a soluble form in sufficient quantities for extraction and purification. Moreover, soluble HCV NS3 protease was not able to be produced in soluble form in bacteria. This is important because bacterial expression is the preferred method of expression of large quantities of HCV protease. Soluble HCV NS3 protease of the present invention can be produced in several ways. A solubilizing motif can be fused to the protein resulting in a soluble protein. A solubilizing motif is any chemical moiety bound to the HCV NS3 protease which results in the NS3 protease becoming soluble in a buffered solution. Examples of such solubilizing motifs are chains of amino acids having polar side chains, preferably positively charged amino acids. The chain of amino acids should be about 4 - 10 amino acid residues in length. The preferred amino acids are arginine and lysine. Another example of a solubilizing motif is an amphipathic moiety. The solubilizing motif can be fused to either the amino terminus or carboxy terminus of the NS3 protease. A sequence which has been successfully fused to the carboxyl terminus to produce soluble NS3 protease is -Arg - Lys - Lys - Lys - Arg - Arg- (SEQ ID NO: 2). This has been fused to the carboxyl end of the NS3 protease to produce the polypeptides of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 8 and SEQ ID NO: 27. Other examples of soluble HCV NS3 protease having a hydrophilic amino acid residue tail which were made are SEQ ID NO: 9, and SEQ ID NO: 10.

Soluble HCV NS3 protease can also be produced which does not have a solubilizing motif as for example the proteases shown in SEQ ID NO: 1 and SEQ ID NO: 7. Preferably the NS3 protease will have a histidine tag fused to its amino acid terminus for use in purifying the protein on a nickel (Ni^{2+}) coated resin. See SEQ ID NO: 5. In this embodiment the protease is produced as insoluble aggregates or as inclusion bodies in bacteria such as in *E. coli*.

The insoluble HCV NS3 protease is first extracted from the bacteria by homogenization or sonication of the bacteria. The aggregates containing the bacteria are then solubilized in a 5 M solution of guanidine hydrochloride (GuHCl). The NS3 protease is then purified

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from high molecular weight aggregates by size exclusion chromatography, as for example by applying the solution to a SEPHACRYL S-300 size exclusion gel column. Fractions containing the NS3 protease in 5 M GuCl are pooled and diluted to about 0.1 M GuHCl in a refolding buffer comprised of dithiothreitol and lauryl maltoside. The diluted solution is then applied to a reverse phase chromatography column and pools containing the NS3 protease collected. The pH of the protease fractions is then raised in a stepwise manner to about 7.4 so as to produce properly refolded soluble, active NS3 protease.

10

It has also been discovered that the HCV NS3 protease is much more effective in cleaving the HCV non-structural proteins, if the co-factor NS4A protein is present (SEQ ID NO: 6). Accordingly, the present invention is also comprised of a fusion of the NS4A cofactor domain protein with the NS3 protease, in particular the fusion of the NS3 protease and the NS4A cofactor wherein the NS4A is mutated such the NS3 protease and the NS4A cofactor is not cleaved by the NS3 protease. Examples of the fused NS3 and NS4A constructs are shown in SEQ ID NOs, 7, 8, 9, 10 and 27.

20

DNA encoding the NS3 protease of this invention can be prepared by chemical synthesis using the known nucleic acid sequence [Ratner *et al.*, Nucleic Acids Res. 13:5007 (1985)] and standard methods such as the phosphoramidite solid support method of Matteucci *et al.* [J Am. Chem. Soc. 103:3185 (1981)] or the method of Yoo *et al.* [J. Biol. Chem. 764:17078 (1989)]. See also Glick, Bernard R. and Pasternak, *Molecular Biotechnology* : pages 55 - 63, (ASM Press, Washington, D.C. 1994). The gene encoding the protease can also be obtained using the plasmid disclosed in Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. M., Expression and Identification of Hepatitis C Virus polyprotein Cleavage Products, *J. Virol* 67:1385-1395 (1993). Also, the nucleic acid encoding HCV protease can be isolated, amplified and cloned (from patients infected with the HCV virus). Furthermore, the HCV genome has been disclosed in PCT WO 89/04669 and are available from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD under ATCC accession no. 40394.

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Of course, because of the degeneracy of the genetic code, there are many functionally equivalent nucleic acid sequences that can encode mature human HCV protease as defined herein. Such functionally equivalent sequences, which can readily be prepared
5 using known methods such as chemical synthesis, PCR employing modified primers and site-directed mutagenesis, are within the scope of this invention.

Various expression vectors can be used to express DNA
10 encoding HCV NS3 protease. Conventional vectors used for expression of recombinant proteins used for expression of recombinant proteins in prokaryotic or eukaryotic cells may be used. Preferred vectors include the pcD vectors described by Okayama *et al.*, Mol. Cell. Bio. Vol. 3: 280-289 (1983); and Takebe *et al.*, Mol. Cell. Biol. Vol. 8: 466-472 (1988). Other
15 SV40-based mammalian expression vectors include those disclosed in Kaufman *et al.*, Mol. Cell. Biol. Vol. 2: 1304-1319 (1982) and U.S. Patent No. 4,675,285. These SV40-based vectors are particularly useful in COS7 monkey cells (ATCC No. CRL 1651), as well as in other mammalian cells such as mouse L cells and CHO cells.

20

Standard transfection methods can be used to produce eukaryotic cell lines which express large quantities of the polypeptide. Eukaryotic cell lines include mammalian, yeast and insect cell lines. Exemplary mammalian cell lines include COS-7 cells, mouse L cells and Chinese
25 Hamster Ovary (CHO) cells. See Sambrook *et al.*, *supra* and Ausubel *et al.*, *supra*.

As used herein, the term "transformed bacteria" means bacteria that have been genetically engineered to produce a mammalian protein.
30 Such genetic engineering usually entails the introduction of an expression vector into a bacterium. The expression vector is capable of autonomous replication and protein expression relative to genes in the bacterial genome. Construction of bacterial expression is well known in the art, provided the nucleotide sequence encoding a desired protein is
35 known or otherwise available. For example, DeBoer in U.S. Pat. No. 4,551,433 discloses promoters for use in bacterial expression vectors; Goeddel *et al.* in U.S. Pat. No. 4,601,980 and Riggs, in U.S. Pat. No. 4,431,739 disclose the production of mammalian proteins by *E. coli*

expression systems; and Riggs *supra*, Ferretti *et al. Proc. Natl. Acad. Sci.*83:599 (1986), Sproat *et al., Nucleic Acid Research* 13:2959 (1985) and Mullenbach *et al., J. Biol. Chem* 261:719 (1986) disclose how to construct synthetic genes for expression in bacteria. Many bacterial expression
5 vectors are available commercially and through the American Type Culture Collection (ATCC), Rockville, Maryland.

Insertion of DNA encoding human HCV protease into a vector is easily accomplished when the termini of both the DNA and
10 the vector comprise the same restriction site. If this is not the case, it may be necessary to modify the termini of the DNA and/or vector by digesting back single-stranded DNA overhangs generated by restriction endonuclease cleavage to produce blunt ends, or to achieve the same result by filling in the single-stranded termini with
15 an appropriate DNA polymerase. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. The cleaved vector and the DNA fragments may also be modified if required by
20 homopolymeric tailing.

Many *E. coli*-compatible expression vectors can be used to produce soluble HCV NS3 protease, including but not limited to vectors containing bacterial or bacteriophage promoters such as the *Tac*, *Lac*, *Trp*, *LacUV5*, $l P_r$ and $l P_L$ promoters. Preferably, a vector
25 selected will have expression control sequences that permit regulation of the rate of HCV protease expression. Then, HCV protease production can be regulated to avoid overproduction that could prove toxic to the host cells. Most preferred is a vector comprising, from 5' to 3' (upstream to downstream), a *Tac* promoter,
30 a *lac Iq* repressor gene and DNA encoding mature human HCV protease. The vectors chosen for use in this invention may also encode secretory leaders such as the *ompA* or protein A leader, as long as such leaders are cleaved during post-translational processing to produce mature HCV protease or if the leaders are not cleaved, the
35 leaders do not interfere with the enzymatic activity of the protease.

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Fusion peptides will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, e.g., in Sambrook, et al. (1989) *Molecular Cloning: A Laboratory Manual* (2d ed.), vols. 1-3, Cold Spring Harbor Laboratory; and Ausubel, et al. (eds.) (1993) *Current Protocols in Molecular Biology*, Greene and Wiley, NY. Techniques for synthesis of polypeptides are described, e.g., in Merrifield (1963) *J. Amer. Chem. Soc.* 85:2149-2156; Merrifield (1986) *Science* 232: 341-347; and Stewart et al (1984)., "Solid Phase Peptide Synthesis" (2nd Edition), Pierce Chemical Co., Rockford, IL.; and Atherton, et al. (1989) *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press, Oxford; and Grant (1992) *Synthetic Peptides: A User's Guide*, W.H. Freeman, NY.

15

One can use the NS3 protease, the NS4 cofactor and the peptide substrates, either 4B/5A or 5A/5B, to develop high throughput assays. These can be used to screen for compounds which inhibit proteolytic activity of the protease. One does this by developing techniques for determining whether or not a compound will inhibit the NS3 protease from cleaving the viral substrates. Examples of such synthetic substrates are SEQ ID NOs 16, 17, 18, 19, 20 and 21. If the substrates are not cleaved, the virus cannot replicate. One example of such a high throughput assay is the scintillation proximity assay (SPA). SPA technology involves the use of beads coated with scintillant. Bound to the beads are acceptor molecules such as antibodies, receptors or enzyme substrates which interact with ligands or enzymes in a reversible manner.

For a typical protease assay the substrate peptide is biotinylated at one end and the other end is radiolabelled with low energy emitters such as ^{125}I or ^3H . The labeled substrate is then incubated with the enzyme. Avidin coated SPA beads are then added which bind to the biotin. When the substrate peptide is cleaved by the protease, the radioactive emitter is no longer in proximity to the scintillant bead and no light emission takes place. Inhibitors of the protease will leave the substrate intact and can be identified by the resulting light emission which takes place in their presence.

Another type of protease assay, utilizes the phenomenon of surface plasmon resonance (SPR). A novel, high throughput enzymatic assay utilizing surface plasmon resonance technology has been successfully developed. Using this assay, and a dedicated BIAcore™ instrument, at least 1000 samples per week can be screened for either their enzymatic activity or their inhibitory effects toward the enzymatic activity, in a 96 well plate format. This methodology is readily adaptable to any enzyme-substrate reaction. The advantage of this assay over the SPA assay is that it does not require a radiolabeled peptide substrate.

10

The following examples are included to illustrate the present invention but not to limit it.

15

Examples 1

Production of HCV NS3 Protease

A. Plasmid constructions.

20

Several plasmids were designed and constructed using standard recombinant DNA techniques (Sambrook, Fritsch & Maniatis) to express the HCV protease in *E. coli* (Fig 2-7). All HCV specific sequences originated from the parental plasmid pBRTM/HCV 1-3011 (Grakoui *et al.* 1993). To express the N-terminal 183 amino acid versions of the protease, a stop codon was inserted into the HCV genome using synthetic oligonucleotides (Fig. 3). The plasmids designed to express the N-terminal 246 amino acid residues were generated by the natural NcoI restriction site at the C-terminus.

30

i) Construction of the plasmid pBJ1015 (Figure 2)

The plasmid pBRTM/HCV 1-3011 containing the entire HCV genome (Grakoui A., *et al.*, *J. Virol.* 67: 1385-1395) was digested with the restriction enzymes Sca I and Hpa I and the 7138 bp (base pair) DNA fragment was isolated and cloned to the Sma I site of pSP72 (Promega) to produce the plasmid, pRJ201. The plasmid pRJ 201 was digested with Msc I and the 2106 bp Msc I fragment was isolated and cloned into the

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Sma I site of the plasmid pBD7. The resulting plasmid pMBM48 was digested with Kas I and Nco I, and the 734 bp DNA fragment after blunt ending with Klenow polymerase was isolated and cloned into Nco I digested, klenow polymerase treated pTrc HIS B seq expression plasmid (Invitrogen). The ligation regenerated a Nco I site at the 5' end and Nsi I site at the 3' end of HCV sequence. The plasmid pTHB HCV NS3 was then digested with Nco I and Nsi I, and treated with klenow polymerase and T4 DNA polymerase, to produce a blunt ended 738 bp DNA fragment which was isolated and cloned into Asp I cut, klenow polymerase treated expression plasmid pQE30 (HIV). The resulting plasmid pBJ 1015 expresses HCV NS3 (246 amino acids) protease.

(ii) Construction of the plasmid pTS 56-9 with a stop codon after amino acid 183 (Figure 3)

The plasmid pTHB HCV NS3 was digested with Nco I, treated with klenow polymerase, then digested with Bst Y I; and the DNA fragment containing HCV sequence was isolated and cloned into Sma I and Bgl II digested pSP72. The resulting plasmid pTS 49-27 was then digested with Bgl II and Hpa I and ligated with a double stranded oligonucleotide:

GA TCA CCG GTC TAG ATCT

T GGC CAG ATC TAGA (SEQ ID NO 11) to produce pTS 56-9.

Thus, a stop codon was placed directly at the end of DNA encoding the protease catalytic domain of the NS3 protein. This enabled the HCV protease to be expressed independently from the helicase domain of the NS3 protein.

(iii) Construction of the plasmid pJB 1006 Fused with a peptide of positively charged amino acids at the carboxy terminus of NS3 183 (Figure 4).

The plasmid pTS 56-9 was digested with Sph I and Bgl II and the DNA fragment containing HCV sequence was isolated and cloned into a Sph I, Bgl II cut pSP72. The resulting plasmid pJB 1002 digested with Age I and HpaI and ligated to a double stranded oligonucleotide,

CCG GTC CGG AAG AAA AAG AGA CGC TAG C

AG GCC TTC TTT TTC TCT GCG ATC G

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(SEQ ID NO 12), to construct pJB 1006. This fused the hydrophilic, solubilizing motif onto the NS3 protease.

- 5 (iv) Construction of the plasmid pBJ 1022 expressing His-NS3(183)-HT in *E.coli* (Figure 5)

The plasmid pJB 1006 was digested with NgoM I and Nhe I and the 216 bp DNA fragment was isolated and cloned into Ngo M I, Nhe I cut pBJ
10 1015 to construct plasmid pBJ 1019. The plasmid pBJ 1019 was digested with Nar I and Pvu II, and treated with Klenow polymerase to fill in 5' ends of Nar I fragments. The expression plasmid pQE31 (Invitrogen) was digested with BamH I, blunt ended with Klenow polymerase. The 717 bp
15 Nar I- Pvu II DNA fragment was isolated and ligated to the 2787 bp BamH I/Klenowed -Msc I (Bal I) fragment of the expression plasmid pQE31 (Invitrogen). The recombinant plasmid, pBJ 1022, obtained after transformation into *E.coli* expresses His NS3(2-183)-HT which does not contain any HIV protease cleavage site sequence. The plasmid also contains a large deletion in the CAT (Chloramphenicol Acetyl
20 Transferase) gene.

- (v) Construction of the plasmid pNB(-V)182-Δ4A HT (Figure 6)

The plasmid pMBM 48 was digested with Eag I and Xho I, treated with
25 Klenow polymerase and the 320 bp DNA fragment was isolated and cloned into BamH I cut, blunt ended pSP 72 to construct the plasmid pJB1004. The 320 bp fragment encodes 7 amino acid from carboxy terminal of NS3(631), all of NS4A, and the amino terminal 46 amino acid of NS4B. The recombinant plasmid pJB1004 was digested with Eag I
30 and Cel 2, blunt ended with Klenow polymerase. The 220 bp DNA fragment was isolated and cloned into the expression plasmid pQE30 which was digested with BamH I and blunt ended with Klenow polymerase prior to ligation. The resulting plasmid pJB 1011 was digested with NgoM I and Hind III and ligated to a double stranded
35 oligonucleotide ,

```
CCG GCA  ATT ATA CCT GAC AGG GAC GTT CTC TAC CAG GAA TTC
      GT  TAA TAT GGA CTG TCC CTA CAA GAG ATG GTC CTT AAG
```

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GAT GAG ATG GAA GAG TGC CGG AAG AAA AAG AGA CGC A
 CTA CTC TAC CTT CTC ACG GCC TTC TTT TTC TCT GCG TTC GA
 (SEQ ID NO 13)

5

to construct the plasmid pNB 4A HT. The plasmid pNB 4AHT was digested with Msl I and Xba I. The 1218 bp DNA fragment was isolated and cloned into Age I cut, klenow polymerase treated, Xba I cut vector DNA of pBJ 1019. The ligation results in a substitution of the 183rd amino acid residue valine by a glycine residue in NS3, and a deletion of amino terminal three amino acid residues of NS4A at the junction. The recombinant plasmid pNB182Δ4A HT comprising NS3(182aa)-G-NS4A(4-54 amino acid) does not contain NS3/NS4A cleavage site sequence at the junction and is not cleaved by the autocatalytic activity of NS3. Finally the plasmid pNB182Δ4A HT (SEQ ID NO 8) was digested with Stu I and Nhe I, the 803 bp DNA fragment was isolated and cloned into Stu I and Nhe I cut plasmid pBJ 1022. The resulting plasmid pNB(-V)182-Δ4A HT contains a deletion of the HIV sequence from the amino terminus end of the NS3 sequence and in the CAT gene (SEQ ID NO 27).

20

(vi) Construction of the plasmid pT5 His HIV-NS3 (Figure 7)

The plasmid pTS56-9 was digested with Bgl II, and treated with Klenow polymerase to fill in 5' ends. The plasmid was then digested with NgoM I and the blunt ended Bgl II/NgoMI fragment containing the NS3 sequence was isolated and ligated to the SglI, Klenow treated NgmMI cut and Sal I klenowed pBJ 1015. The resulting plasmid is designated pT5His HIV 183.

25

30

Example 2

Purification of HCV NS3 Protease having a Solubilizing Motif

Purification of His182HT (SEQ ID NO 4) and

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His (-V)182Δ4AHT (SEQ ID NO 8)

The recombinant plasmids pBJ1022 and pNB(-V)182Δ4A were used to transform separate cultures of *E. coli* strain M15 [pREP4]

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(Qiagen), which over-expresses the *lac* repressor, according to methods recommended by the manufacturer. M15 [pREP4] bacteria harboring recombinant plasmids were grown overnight in broth containing 20g/L bactotrypton, 10g/L bacto-yeast extract, 5g/L NaCl (20-10-5 broth) and supplemented with 100µg/ml ampicillin and 25µg/ml kanamycin. Cultures were diluted down to O.D.600 of 0.1, then grown at 30°C to O.D.600 of 0.6 to 0.8, after which IPTG was added to a final concentration of 1mM. At post-induction 2 to 3 hours, the cells were harvested by pelleting, and the cell pellets were washed with 100mM Tris, pH 7.5. Cell lysates were prepared as follows: to each ml equivalent of pelleted fermentation broth was added 50µl sonication buffer (50mM sodium phosphate, pH 7.8, 0.3M NaCl) with 1mg/ml lysozyme; cell suspension was placed on ice for 30 min. Suspension was then brought to a final concentration of 0.2% Tween-20, 10mM dithiothreitol (DTT), and sonicated until cell breakage was complete. Insoluble material was pelleted at 12,000 x g in a microcentrifuge for 15 minutes, the soluble portion was removed to a separate tube and the soluble lysate was then brought to a final concentration of 10% glycerol. Soluble lysates from cells expressing the plasmids produce strongly immunoreactive bands of the predicted molecular weight. Soluble lysates prepared for Ni²⁺ column purification were prepared with 10mM β-mercaptoethanol (BME) instead of DTT. Lysates were stored at -80°C .

25 Purification using Ni²⁺-Nitrosyl acetic acid (NTA) agarose (QIAGEN)

The proteins were then purified by placing the extracted lysate on an NTA agarose column. NTA agarose column chromatography was used because the histidine tag which was fused to the N-terminus of the proteases readily binds to the nickel column. This produces a powerful affinity chromatographic technique for rapidly purifying the soluble protease. The column chromatography was performed in a batch mode. The Ni²⁺ NTA resin (3ml) was washed twice with 50 ml of Buffer A (50mM sodium phosphate pH 7.8 containing 10% glycerol, 0.2% Tween-20, 10mM BME). The lysate obtained from a 250 ml fermentation (12.5 ml) was incubated with the resin for one hour at 4°C. The flow through was collected by centrifugation. The resin was packed into a 1.0 x 4 cm column and washed with buffer A until the baseline was reached. The

- 19 -

bound protein was then eluted with a 20 ml gradient of imidazole (0-0.5M) in buffer A. Eluted fractions were evaluated by SDS-PAGE and western blot analysis using a rabbit polyclonal antibody to His-HIV 183.

5 Purification using POROS metal-chelate affinity column

In an alternative method to purify the proteins the lysate containing the proteins were applied to a POROS metal-chelate affinity column. Perfusion chromatography was performed on a POROS MC metal
10 chelate column (4.6 x 50mm, 1.7 ml) precharged with Ni²⁺. The sample was applied at 10 ml/min and the column was washed with buffer A. The column was step eluted with ten column volumes of buffer A containing 25 mM imidazole. The column was further eluted with a 25
15 column volume gradient of 25-250 mM imidazole in buffer A. All eluted fractions were evaluated by SDS-PAGE and western blot analysis using rabbit polyclonal antibody.

20

Example 3

Peptide Synthesis of the 5A/5B and 4B/5A Substrates

The peptides 5A/5B and 4B/5A substrates (SEQ ID NOs 16, 18, 19, 20 and
25 21) were synthesized using Fmoc chemistry on an ABI model 431A peptide synthesizer. The manufacture recommended FastMoc™ activation strategy (HBTU/HOBt) was used for the synthesis of 4A activator peptide. A more powerful activator, HATU with or without the additive HOAt were employed to assemble 5A/5B substrate peptides
30 on a preloaded Wang resin. The peptides were cleaved off the resin and deprotected by standard TFA cleavage protocol. The peptides were purified on reverse phase HPLC and confirmed by mass spectrometric analysis.

35

Example 4**HPLC-assay using a synthetic 5A/5B peptide substrate**

- 5 To test the proteolytic activity of the HCV NS3 protease the
DTEDVVCC SMSYTWGK (SEQ ID NO 16) and soluble HCV NS3 (SEQ
ID NO 27) were placed together in an assay buffer. The assay buffer was
50mM sodium phosphate pH 7.8, containing 15% glycerol, 10mM DTT,
0.2% Tween20 and 200 mM NaCl). The protease activity of SEQ ID NO
10 27 cleaved the substrate into two byproduct peptides, namely 5A and 5B.
The substrate and two byproduct peptides were separated on a reversed-
phase HPLC column. (Dynamax, 4.6 x 250 mm) with a pore size of 300Å
and a particle size of 5µm. The column was equilibrated with 0.1%TFA
(Solvent A) at a flow rate of 1 ml per minute. The substrate and the
15 product peptide standards were applied to the column equilibrated in A.
Elution was performed with a acetonitrile gradient (Solvent B=100%
acetonitrile in A). Two gradients were used for elution (5% to 70%B in
50 minutes followed by 70% to 100%B in 10 minutes).
- 20 In another experiment, partially purified SEQ ID NO 27 or vector
control was incubated with 100µM of substrate for 3, 7 and 24 hours at
30°C. The reaction mixture was quenched by the addition of TFA to
0.01% and applied to the reversed-phase HPLC column. The fractions
from each run were evaluated by mass spectrometry and sequencing.
25

Example 5**30 Analysis of NS3 Protease Activity By *In Vitro* Translation Assay**

- To detect HCV NS3 protease activity in *trans*, we have expressed a
40 kD protein containing the NS5A/5B cleavage site in cell-free
translation system and used that as the substrate for the enzyme. The
35 substrate protein produces two protein products of apparent molecular
weight 12.5 kD (NS 5A') and 27 kD (NS5B') upon cleavage by the HCV
NS3 protease.

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The plasmid pTS102 encoding the substrate 5A/5B was linearized by digestion with EcoR I and was transcribed using T7 RNA polymerase *in vitro*. The RNA was translated in presence of ³⁵S methionine in rabbit reticulocyte lysates according to the manufacturer's (Promega) protocol to produce HCV specific protein. In a 20 µl total reaction mixture containing 10mM Tris, pH 7.5, 1mM DTT, 0.5mM EDTA, and 10% glycerol was placed 2 to 8 µl of ³⁵S methionine-labeled translated 5A/5B substrate. The reaction was started with the addition of 10µl of HCV NS3 protease in solubilization buffer (50mM Na Phosphate, pH 7.8, 0.3M NaCl, 0.2% Tween 20, 10 mM DTT or BME, 10% glycerol), and incubated at 30°C for the specified time. Reactions were stopped by adding an equal volume of 2X Laemmli sample buffer (Enprotech Inc.) and heating at 100°C for 3 minutes. Reaction products were separated by SDS PAGE electrophoresis; gels were fixed, dried and subjected to autoradiography.

The *in vitro* translated substrate was used to assay the HCV NS3 proteases expressed by *E. coli* harboring plasmids pBJ1022 and pNB(-V)182Δ4A (SEQ ID NOs 4 and 27). In a two hour assay incubated at 30°C, pBJ1022 crude soluble lysate at 3, 6, and 10µl, was able to cleave 5A/5B substrate in a dose responsive manner, producing the expected cleaved products: 5A (12.5 kD) and 5B (27 kD) as shown by SDS PAGE analysis. Corresponding vector control lysate did not show any cleavage activity over background. The crude soluble lysate derived from pNB182Δ4A was much more active in this assay. After only 30 minutes incubation, the 5A and 5B cleavage products were detected using as little as 0.125µl cell lysate, with increasing amounts of lysate showing increased cleavage, reaching a maximum at 1µl.

We performed a time course study of the NS3 Protease activity of pNB182Δ4A in an *in vitro* translation assay for further characterization of the activity. At 30°C, in a reaction containing the translated 5A/5B substrate plus pNB182Δ4A soluble lysate at 1µl per 20µl reaction volume, the 5A and 5B cleavage products appeared beginning at 1 minute, and increased with time at 2.5, 5, 10, and 20 minutes.

Since we were able to demonstrate HCV NS3 Protease activity using crude cell lysates of pBJ1022 and pNB182Δ4A, we wanted to at least

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partially purify the expressed proteins in an effort to remove bacterial proteases from these preparations. For this purpose, affinity column chromatography using Ni^{2+} bound ligands was found to be effective, binding the histidine tag at the amino terminal ends of the expressed proteins, and subsequently releasing the bound proteins by imidazole elution. The imidazole-eluted fractions resulting from the purification of pNB182Δ4A on a Ni-NTA column were tested for activity in the *in vitro* translation assay. The resultant fractions were all able to cleave the translated 5A/5B Substrate, producing the expected 5A and 5B products. Background bacterial protease activity was not detected in these eluted fractions .

As was described above, pBJ1022 was purified by another method of Ni^{2+} chelate chromatography, using POROS Ni^{2+} chelate resin and perfusion chromatography. Imidazole-eluted fractions which were positive for immunoreactivity with antibody to NS3 183 were tested for HCV protease activity by *in vitro* translation assay. In order to optimize detection of activity in this assay for HCV protease, reactions were supplemented with a truncated peptide derived from the NS4A cofactor which has been shown to enhance cleavage at the 5A/5B site by NS3 protease . The cofactor was supplied as a synthetic peptide containing amino acids 22 to 54 of NS4A (strain HCV-BK) at a final concentration of $1\mu\text{M}$. All fractions tested were active in this translation assay.

25

Example 6

ENHANCEMENT BY 4A PEPTIDES

NS4A is able to enhance the NS3 serine protease activity at NS5A/5B site in mammalian cells that transiently coexpress NS3, NS4A, and the various HCV non-structural polyprotein containing downstream cleavage sites . We have studied this enhancement activity in a well defined cell-free biochemical assay, using the partially purified *E.coli*-expressed pBJ1022 as a source of NS3 protease, and synthetic peptides containing various truncations of NS4A. In our first experiment we used a crude cell lysate of pBJ1022 as the enzyme and NS4A synthetic peptide truncated 33 mer from amino acid 22 to amino

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acid 54, the carboxy-terminal *in vitro* translation cleavage reaction .

The C-terminal 33 amino acid peptide of NS4A was able to enhance the activity of the NS3 catalytic domain in a dose dependent manner from 0.01 μ M to 1.0 μ M peptide, producing the expected products of 5A

5 (12.5kD), and 5B (27kD) from the 40kD translated 5A/5B substrate.

Without the 4A peptide a relatively low cleavage activity by the protease alone was observed at the short incubation time of 30 minutes. The 4A peptide itself or with the combination of crude lysate produced from cells harboring the vector plasmid did not cleave the substrate.

10

To further characterize NS4A enhancement activity additional truncations were made to the NS4A sequence. Truncated peptides were evaluated for their activity in the *in vitro* translation assay using Ni²⁺ chelate column-purified pBJ1022 (NS3 catalytic domain). We observed
15 that in addition to the C-terminal 33 amino acid peptide, a 18 amino acid peptide containing the NS4A sequence from amino acid 19 through 36 was able to enhance the NS3 mediated cleavage activity. Other peptides, including the N-terminal 21 amino acid, and two shorter truncations from the carboxyl terminal end, a 22mer and a 15mer, were found to
20 have no effect; also a heterologous peptide of 18 amino acid also had no enhancement activity.

Discussion

25 The experiments described in this report clearly demonstrate that bacterially expressed HCV protease catalyzes cleavage of i) HCV polyproteins and ii) synthetic peptide substrates in *trans* biochemical assay. The processing activity of NS3 catalytic domain is enhanced by NS4A and its derivatives. The activity of the fusion protein containing
30 the NS3 catalytic domain and NS4A is much superior to that of the NS3 catalytic domain alone.

Hydrophobicity analysis of the catalytic domain of the NS3 protease reveals that the protein is very hydrophobic and also it contains
35 seven cysteine residues. To neutralize hydrophobicity and thus to improve solubility we have added six positively charged amino acid residues as a solubilizing motif. The addition of a solubilizing motif

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appears to improve the solubility without affecting the enzymatic activity.

5 We have also shown that the HCV NS4A from Japanese BK strain has enhanced the HCV-H NS3 mediated cleavage at 5A/5B site. This suggests that essential elements of recognition may be conserved among various strains of HCV.

10 It is clear from above experimental results that attachment of hydrophilic tail (solubilizing motif/water attracting structures) at the carboxy terminal end of histidine fused NS3 catalytic domain improved expression of soluble protein in *E.coli*. In these experiments six residues of positively charged amino acids are attached at the carboxy terminal end of the protein. It should be noted that other fusions that contained
15 six histidine residues, GST (Glutathione S transferase), MBP (Maltose binding protein), thioredoxin alone did not show improved solubility of NS3. Other examples of solubilizing motif are amphipathic helix tail (peptides having charged and hydrophobic amino acid residues to form both charged and hydrophobic faces). Addition of an amphipathic helix
20 at the carboxy terminus of such fusion proteins will be an alternative way to achieve improvement of solubility without affecting the enzymatic activity.

25 The hydrophilic tail used in these experiments consists of six amino acids. The sequence and length of the hydrophilic amino acids can be varied to achieve optimal expression of soluble protein. Therefore size of the solubilizing motif and nature of charged residues may effect the expression of soluble NS3 in *E.coli*.

30 Position of these water attracting structures/motifs at both ends, at one end (amino terminal or carboxy terminal), or insertion within the NS3 catalytic domain and NS3 (catalytic domain)-4A fusion protein, may improve solubility of the protein without affecting the activity.

35 Based on sequence homology to the members of trypsin superfamily and the protease of other members of the flaviviruses, it is predicted that the amino terminal 181 amino acid of NS3 is the catalytic domain of HCV NS3 protease. Recently it has also been shown that a

- 25 -

protein of 169 amino acid containing a 10 amino acid deletion from the amino terminus and 2 amino acid from carboxy terminal of the catalytic domain retains full enzymatic activity. The model we have developed predicts that a protein of 154 amino acids containing a deletion of 26
5 amino acid from amino terminal and a deletion of 2 amino acid from the carboxyl terminus would retain full enzymatic activity for the 5A/5B substrate.

Analysis of the amino acid sequence of the catalytic domain of
10 NS3 protease reveals that the protein contains seven cysteine residues, an odd number, which may cause aggregation. Mutation of one cysteine residue (located on the surface of the protein molecule and not involved in the activity) may improve solubility of the protein without affecting the protease activity.

15

Using the cell free biochemical assay we have demonstrated that the synthetic peptide containing 18 amino acid of HCV NS4A protein is sufficient to enhance the cleavage at NS5A/5B site mediated by the catalytic domain of NS3.

20

Example 7

Refolding of Insoluble HCV NS3 Protease

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The present example describes a novel process for the refolding of HCV NS3 protease which does not have a solubilizing motif from an *E.coli* inclusion body pellet. This procedure can be used to generate purified enzyme for activity assays and structural studies.

30

Extraction and Purification of His-HIV 183 from the *E.coli* inclusion body pellet

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E. coli cells harboring the plasmid for HisHIV183 was used to transform a culture of *E. coli* strain M15 [pREP4] (Qiagen), which over-expresses the *lac* repressor, according to methods recommended by commercial source. M15 [pREP4] bacteria harboring recombinant

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plasmids were grown overnight in 20-10-5 broth supplemented with 100µg/ml ampicillin and 25µg/ml kanamycin. Cultures were diluted to O.D.600 of 0.1, then grown at 37°C to O.D.600 of 0.6 to 0.8, after which IPTG was added to a final concentration of 1mM. At post-induction 2 to 3 hours, the cells were harvested by pelleting, and the cell pellets were washed with 100mM Tris, pH 7.5. were pelleted by centrifugation. The cell pellet was resuspended in 10 ml of 0.1M Tris-HCl, 5mM EDTA, pH 8.0 (Buffer A) for each gm wet weight of pellet. The pellet was homogenized and resuspended using a Dounce homogenizer. The suspension was clarified by centrifugation at 20,000 x g for 30 minutes at 4°C. The pellet was sequentially washed with the following five buffers:

1. Buffer A
2. 1.0M sodium chloride (NaCl) in buffer A
3. 1.0% Triton X-100 in buffer A
4. Buffer A
5. 1.0 M Guanidine HCl (GuHCl) in buffer A.

The washed pellet was solubilized with 5M GuHCl, 1% beta mercaptoethanol in buffer A (3 ml per gm wet wt. of pellet) using a Dounce homogenizer and centrifuged at 100,000 x g for 30 minutes at 4°C. Purification of denatured HisHIV183 from high molecular weight aggregates was accomplished by size exclusion on a SEPHACRYL S-300 gel filtration column.

In particular, an 8 ml sample of the 5.0M GuHCl *E. coli* extract was applied to a 160 ml Pharmacia S-300 column (1.6 x 100 cm) at a flow rate of 1.0 ml/min. The column buffer was comprised of 5.0 M GuHCl, 0.1 M Tris-HCl, pH 8.0, and 5.0 mM EDTA. The fraction size was 5.0 ml. Appropriate fractions were pooled based on the results of SDS-PAGE, as well as N-terminal sequence analysis of the protein transferred to a Pro-Blot.

Detergent-assisted refolding of HCV-protease

The protein was concentrated by ultrafiltration using a 43 mm Amicon YM10 membrane to 1.0 mg per ml in 5M GuHCl, 0.1M Tris-HCl

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pH 8.0, 1.0 mM EDTA, 1.0% beta-mercaptoethanol. It was then diluted 50-fold to 0.1M GuHCl in refolding buffer (100 mM sodium phosphate pH 8.0, 10mM DTT, 0.1% lauryl maltoside) and the mixture was incubated on ice for at least one hour. A 25 ml sample containing 500 µg of the protein in the refolding buffer was applied to a Pro-RPC HR 3/5 reversed phase chromatography column. The applied sample contained 500 µg protein in 25 ml of refolding buffer. To the column was then applied a solution B comprised of 99.9% H₂O + 0.1% trifluoroacetic acid (TFA). A 10 ml volume of solution C [10% H₂O, 90% acetonitrile (AcN) + 0.1% TFA] was applied to the column at a 0 - 60% gradient into solution B at a flow rate of 0.5ml/min. and a fraction size of 0.5ml. The fractions were monitored at A214; 2.0 absorbance units full scale (AUFs).

Fractions containing the protein (corresponding to peak 1) were pooled for renaturation by stepwise dialysis. The fractions were first dialysed in 0.1% TFA in 25% glycerol overnight at 4°C ; then dialyzed in 0.01% TFA in 25% glycerol overnight at 4°C; then dialyzed in 0.001% TFA in 25% glycerol for 3.0 hours; then dialyzed for 3 hours at 4°C in 50 mM NaPO₄, pH 6.0, 10 mM dithiotreitol (DTT) in 25% glycerol. The protein was then dialyzed for 3.0 hours at 4°C in 50 mM NaPO₄, pH 7.0, 0.15 M NaCl, 10 mM DTT in 25% glycerol; and then finally dialyzed in 50 mM NaPO₄, pH 7.8, 0.3 M NaCl, 10 mM DTT, 0.2% Tween 20 in 25% glycerol. This resulted in purified, refolded, soluble, active HCV NS3 protease.

25

Far UV circular dichroism (CD) analysis of the protein was used to monitor the refolding from an acid denatured state to a folded state at neutral pH. The protein recovery was monitored by a UV scan and SDS-PAGE analysis.

30

Results:

Detergent-assisted Refolding of His-HIV183

35

HisHIV183 was quantitatively extracted from an *E. coli* inclusion body pellet. SDS-PAGE analysis at the various stages of extraction shows that sequential washes are essential to remove significant amounts of the contaminating proteins. HisHIV183 was extracted from the washed

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inclusion body pellet in the presence of 5M GuHCl. The 5M GuHCl extract was applied to a SEPHACRYL S-300 column and the appropriate fractions were pooled based on SDS-PAGE analysis. The amino acid sequence of the first ten residues was verified.

5

Refolding was performed at very low concentrations of protein, in the presence of DTT, lauryl maltoside and glycerol at 4°C. The diluted protein was concentrated on a Pro-RPC reversed phase column. Two peaks were obtained based on the UV and protein profile. Only Peak 1
10 has yielded soluble protein after stepwise dialysis. Far UV CD spectral analysis was used to monitor refolding from a denatured state at acid pH to a folded state at neutral pH. At pH 7.4, the protein was found to exhibit significant amounts of secondary structure that is consistent with that of beta sheet protein. At low pH, the CD spectrum showed that it is
15 fully random coil, having a minimal molar ellipticity at 200nm. The ratio of this minimum at 200nm to that of the shoulder at 220 nm is approximately 4:1. This ratio decreased when the secondary structure formation occurred at neutral pH.

20

A UV scan at each step of dialysis showed that the protein recovery was >90% up to pH 7.4 and that there was no light scattering effect due to protein aggregates. SDS-PAGE analysis also indicated that there was no loss of protein up to pH 7.0 during refolding. Precipitation of protein occurred at the last step of dialysis, and the soluble protein
25 was clarified by centrifugation. The overall protein recovery was about 0.10%. The refolded protein was found to be active in a trans-cleavage assay using the *in vitro*-translated 5A/5B substrate in the presence of 4A peptide as described in the next example.

30

Example 8

Analysis of Refolded NS3 Protease Activity by In Vitro Translation Assay

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To detect HCV NS3 protease activity in *trans*, we have expressed a 40 kD protein containing the NS5A/5B cleavage site in cell-free translation system and used that as the substrate for the enzyme.

- 29 -

The substrate protein produces two protein products of apparent molecular weight 12.5 kD (NS 5A') and 27 kD (NS5B') upon cleavage by the HCV NS3 protease.

5 The plasmid pTS102 encoding the substrate 5A/5B was linearized by digestion with EcoR I and was transcribed using T7 RNA polymerase *in vitro*. The RNA was translated in presence of ³⁵S methionine in rabbit reticulocyte lysates according to the manufacturer's (Promega) protocol to produce HCV specific protein. In a 20 µl total
10 reaction mixture containing 10mM Tris, pH 7.5, 1mM DTT, 0.5mM EDTA, and 10% glycerol was placed 2 to 8 µl of ³⁵S methionine-labeled translated 5A/5B substrate. The reaction was started with the addition of 10µl of HCV NS3 protease (SEQ ID NO: 5) with an approximately equimolar amount (2 µM) of the carboxyterminal 33 mer cofactor NS4A
15 (SEQ ID NO: 29) in solubilization buffer (50mM Na Phosphate, pH 7.8, 0.3M NaCl, 0.2% Tween 20, 10 mM DTT or BME, 10% glycerol), and incubated at 30°C for about one hour. Reactions were stopped by adding an equal volume of 2X Laemmli sample buffer (Enprotech Inc.) and heating at 100°C for 3 minutes. Reaction products were separated by SDS
20 PAGE electrophoresis; gels were fixed, dried and subjected to autoradiography.

The assay was able to cleave 5A/5B substrate in a dose responsive manner, producing the expected cleaved products: 5A
25 (12.5 kD) and 5B (27 kD) as shown by SDS PAGE analysis. The production of cleaved 5A and 5B polypeptides from the 5A/5B substrate is proof that soluble, active, refolded HCV protease was indeed produced by the process of example 7.

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Example 9

Surface Plasmon Resonance Assay

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The present example illustrates a method for determining if a compound can be useful as an HCV protease inhibitor using the surface plasmon resonance assay. Figures 8A and 8B illustrate the technique.

- 30 -

BIAcore™ is a processing unit for Biospecific Interaction Analysis. The processing unit integrates an optical detection system with an autosampler and a microfluidic system. BIAcore™ uses the optical phenomena, surface plasmon resonance to monitor interaction between biomolecules. SPR is a resonance phenomenon between incoming photons and electrons on the surface of thin metal film. Resonance occurs at a sharply defined angle of incident light. At this angle, called the resonance angle, energy is transferred to the electrons in the metal film, resulting in a decreased intensity of the reflected light. SPR response depends on a change in refractive index in the close vicinity of the sensor chip surface, and is proportional to the mass of analyte bound to the surface. BIAcore continuously measure the resonance angle by a relative scale of resonance units (RU) and displays it as an SPR signal in a sensorgram, where RU are plotted as a function of time.

In addition, BIAcore™ uses continuous flow technology. One interactant is immobilized irreversibly on the sensor chip, comprising a non-crosslinked carboxymethylated dextran providing a hydrophilic environment for bimolecular interaction. Solution containing the other interactant flow continuously over the sensor chip surface. As molecules from the solution bind to the immobilized ligand, the resonance angle changes resulting in a signal registered by the instrument.

In this methodology, the enzymatic reactions are carried out outside of the BIAcore, i.e. in reaction tubes or 96-well tissue culture plates; as it is conventionally done for any of the currently available high throughput assays. The SPR is only used as a detection means for determination of the amount of an intact substrate remaining in a solution with and without the enzyme after the reaction is quenched.

In order to measure the amount of the intact substrate prior to the addition of enzyme, a means of capturing the substrate onto the sensor chip had to be established. In addition, to satisfy the requirement for a high throughput assay on the BIAcore, the substrate needed to be removed from the surface subsequent to completion of analysis. This is

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required since the same surface will be used for the subsequent reactions. To accomplish these two requirements, a phosphotyrosine is synthetically attached to one end of the substrate. The phosphotyrosine was chosen due to the commercial availability of an anti-
5 phosphotyrosine monoclonal antibody. The antibody is covalently attached to the sensor chip by standard amine coupling chemistry. The anti-phosphotyrosine antibody, bound permanently to the chip is used to capture the phosphotyrosine-containing substrate in a reversible manner. The antibody-phosphotyrosine interaction is ultimately used
10 to capture and release the peptide substrate when desired by regeneration of the surface with various reagents i.e. 2 M $MgCl_2$.

Introduction of the intact peptide onto the antibody surface results in a larger mass which is detected by the instrument. To follow
15 the extent of peptide cleavage, a mixture of peptide substrate and enzyme is incubated for the desired time and then quenched. Introduction of this mixture containing the cleaved peptide and the intact peptide to a regenerated antibody surface results in a lower mass value than that detected for a sample containing only intact peptide.
20 The difference in the two values is then used to calculate the exact amount of intact peptide remaining after cleavage by the enzyme.

Although the reduction in mass can be directly followed with many large substrates, due to the small mass of a typical synthetic
25 peptide substrate (10-20 amino acids, 1-3 Daltons), the mass difference, and thus the signal difference between the intact and cleaved peptide is very small within the signal to noise ratio of the instrument. To circumvent this low sensitivity, we attached a biotin at the N-terminus of the peptide. By addition and thus tagging of peptide with streptavidin
30 prior to injection of tagged peptide onto the antibody surface of the chip, the signal due to the presence of streptavidin will be higher. Using this approach, a cleaved peptide lacking the N-terminal half, tagged with streptavidin will result in a much lower signal.

35 The HCV protease 5A-5B peptide substrate, DTEDVVACSMSYTWGK (SEQ ID NO 18) was synthesized with an additional phosphotyrosine at the C-terminus and biotin at the N-terminus. The biotin was then tagged with streptavidin. An anti-

- 32 -

phosphotyrosine monoclonal antibody, 4G10 (Upstate Biotechnology Inc., Lake Placid, New York) was coupled to the sensor chip. In the absence of HCV protease, the intact, streptavidin-tagged biotinylated phosphotyrosine peptide results in a large signal (large mass unit/large signal) through its interaction with the anti-phosphotyrosine monoclonal antibody (Mab).

The protease-catalyzed hydrolysis of the phosphotyrosine-biotinylated peptide was carried out in a 96 well plate. The reaction was stopped with an equal volume of mercuribenzoate. The cleaved peptide which lacks the tagged streptavidin (less mass) results in the loss of response units (lower signal).

Using this method, numerous compounds can be tested for their inhibitory activity since the antibody surface can be regenerated repetitively with 2 M MgCl_2 .

Procedure for Coupling Anti-phosphotyrosine Mab to the Sensor Chip

The anti-phosphotyrosine Mab is coupled to the carboxymethylated dextran surface of a sensor chip in the following manner. The flow rate used throughout the coupling procedure is 5 $\mu\text{l}/\text{min}$. The surface is first activated with a 35 μl injection of NHS/EDC (N-hydroxysuccinimide/N-dimethylaminopropyl-N'-ethylcarbodiimide-HCl). This is followed by a 40 μl injection of Mab 4G10 at 50 $\mu\text{g}/\text{ml}$ in 10 mM sodium acetate buffer, $\text{pH}=4.0$. Any remaining activated esters are then blocked by the injection of 35 μl of 1 M ethanolamine. These conditions result in the immobilization of approximately 7,500 response units (420 μM) of antibody.

Binding of Peptide and Regeneration of Mab 4G10 Surface

The flow rate used throughout the BIAcore analysis run is 5 $\mu\text{l}/\text{min}$. A 4 μl injection containing streptavidin-tagged peptide (peptide concentration at 2 μM , streptavidin binding sites concentration at 9 μM) is carried out. The amount of streptavidin-tagged peptide bound to the antibody surface (in response units) is measured 30 seconds after the injection is complete.

- 33 -

Regeneration of sensor chip surface

5 Regeneration of the Mab 4G10 surface is achieved using a 4 μ l pulse of 2 M $MgCl_2$ after each peptide injection. Surfaces regenerated up to 500 times still showed 100% binding of tagged peptide.

Determination of the Optimal Concentration of Peptide and Streptavidin

10

To determine the optimal peptide concentration, a standard curve was generated using various amounts of peptide (0-10 μ M) in the presence of excess streptavidin. A value in the linear range, 2 μ M, was chosen for standard assay conditions.

15

The amount of streptavidin required to completely tag the peptide was determined using a peptide concentration of 2.5 μ M and titrating the amount of streptavidin (μ M of binding sites). All the peptides were shown to be completely tagged when streptavidin concentrations greater than 3 μ M (approximately equimolar to the peptide concentration) were used. A streptavidin concentration of 9 μ M (a 4.5 fold excess) was chosen for standard assay conditions.

25

Application of Described Methodology to HCV Protease

The HCV protease 5A/5B peptide substrate, DTEDVVACSMSYTWTKG (SEQ ID NO 18), with phosphotyrosine at the C-terminal and biotin at the N-terminal is synthesized. Anti-phosphotyrosine monoclonal antibody, 4G10 was coupled to the sensor chip.

35 In the absence of HCV protease, the intact streptavidin-tagged biotinylated phosphotyrosine peptide results in a large signal (large mass unit/large response units) through its interaction with the anti-phosphotyrosine monoclonal antibody.

- 34 -

The protease-catalyzed hydrolysis of the phosphotyrosine-biotinylated peptide was carried out in a 96 well plate. The reaction was stopped with an equal volume of the quenching buffer containing mercuribenzoate. Streptavidin was added to tag the peptide which binds to the biotin. The cleaved peptide which lacks the tagged streptavidin (less mass) results in the loss of response units.

Using this assay, numerous compounds can be tested for their inhibitory activity since the antibody surface can be regenerated repetitively with 2 M $MgCl_2$.

The peptide cleavage activity by HCV protease can be monitored in a time dependent manner using the BIAcore-based methodology. Using the concentrated enzyme and the BIAcore substrate, Biotin-DTEDVVAC SMSYTWGK-pY (SEQ ID NO 17), 50% substrate cleavage is achieved within 1 hour using the BIAcore-based HCV assay. Based on the amount of enzyme, His-NS3(183) Δ 4AHT needed to reach a 50% cleavage within 2 hours, a time scale desired for a development of a high throughput assay, we estimate that 1 liter of fermentation of the His-NS3(183) Δ 4AHT construct results in enough protease to run at least 100 reactions on the BIAcore.

Standard Operating Procedure for BIAcore-based HCV Assay

Reactions are prepared in a 96-well tissue culture plate using the Reaction Buffer (50 mM HEPES, pH 7.4, 20 % glycerol, 150 mM NaCl, 1mM EDTA, 0.1% Tween-20, 1 mM DTT) as diluent. The final reaction volume is 100 μ l. Sample with the peptide alone (Biotin-DTEDVVAC SMSYTWGKpY) is prepared by addition of 10 μ l of peptide stock at 100 μ M (prepared in the reaction buffer) to 90 μ l of reaction buffer, so that the final concentration of peptide is 10 μ M. Samples comprised of peptide and the enzyme are prepared by addition of 10 μ l of peptide stock at 100 μ M and 10 μ l of partially purified His-NS3 (183)- Δ 4A-HT stock at 1.7 mg/ml (both prepared in the reaction buffer) to 80 μ l of reaction buffer, so that the final concentration of peptide and the enzyme is 10 and 0.1 μ M respectively. The reaction is held at 30°C for the specified time and then quenched. Quenching is achieved by transferring a 20- μ l aliquot of the reaction mixture to a new tissue

- 35 -

culture plate containing an equal volume of PMB Quenching Buffer (50 mM HEPES, pH 7.8, 150 mM NaCl, 5 mM *p*-Hydroxymercuribenzoic Acid, and 13 mM EDTA).

- 5 To prepare the quenched reaction mixture for injection onto the sensor surface, 30 μ l PMB BIAcore Buffer (50 mM HEPES, pH 7.4, 1 M NaCl) and 30 μ l of streptavidin at 0.5 mg/ml in water is added to the 40 μ l of the quenched reaction mixture to a final volume of 100 μ l. In this step, all the peptides are tagged with streptavidin prior to the injection
- 10 of samples. Finally, 4 μ l of this sample is injected over the antiphosphotyrosin surface for determination of the intact versus cleaved peptide. The final concentration of peptide and the streptavidin in the BIAcore sample is 2 and 9 μ M respectively.

15 Experimental Conditions:

20	<u>Substrate:</u> ID	<u>Biotin-DTEDVVAC SMSYTWTKGK-pY</u> (SEQ NO 19) in Reaction buffer without DTT
	<u>Concentration:</u>	170 μ M (Crude peptide, based on weight)
	<u>Enzyme:</u>	10 μ l of concentrated His-NS3 (183)- Δ 4A-HT at 1.7 mg/ml
25	<u>Reaction volume:</u>	100 μ l
	<u>Reaction buffer:</u>	50 mM HEPES, pH 7.8 20 % glycerol 150 mM NaCl 1mM EDTA 1mM DTT 0.1% Tween-20
30		
35	<u>Temp:</u>	30° C
	<u>Quench with:</u>	<i>p</i> -hydroxymercuribenzoate

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SEQUENCE LISTING

5

(1) GENERAL INFORMATION:

(i) APPLICANT: Schering Corporation

10

(ii) TITLE OF INVENTION: Soluble, Cleavable Substrates of the
Hepatitis C Protease

(iii) NUMBER OF SEQUENCES: 31

15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Schering Corp.

(B) STREET: 2000 Galloping Hill Road

(C) CITY: Kenilworth

20

(D) STATE: New Jersey

(E) COUNTRY: USA

(F) ZIP: 07033-0530

(v) COMPUTER READABLE FORM:

25

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: Apple Macintosh

(C) OPERATING SYSTEM: Macintosh 7.1

(D) SOFTWARE: Microsoft Word 5.1a

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

35

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/439,747

(B) FILING DATE: 12-MAY-1995

(viii) ATTORNEY/AGENT INFORMATION:

- 37 -

(A) NAME: Lunn, Paul G.

(B) REGISTRATION NUMBER: 32,743

(C) REFERENCE/DOCKET NUMBER: JB0509PCT

5

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 908-298-5061

(B) TELEFAX: 908-298-5388

10 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 549 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20 (ix) FEATURE:

(A) NAME/KEY: HCV NS3 Protease

25 GCG CCC ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG 45
Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly
1 5 10 15

TGT ATA ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG 90
30 Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu
20 25 30

GGT GAG GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA 135
Gly Glu Val Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala
35 40 45

- 38 -

ACG TGC ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA 180
 Thr Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly
 50 55 60

5
 ACG AGG ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT 225
 Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr
 65 70 75

10
 ACC AAT GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT 270
 Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly
 80 85 90

15
 TCC CGC TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC 315
 Ser Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr
 95 100 105

20
 CTG GTT ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT 360
 Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly
 110 115 120

25
 GAT AGC AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA 405
 Asp Ser Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu
 125 130 135

30
 AAA GGC TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC 450
 Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala
 140 145 150

35
 GTG GGC CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG 495
 Val Gly Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys
 155 160 165

GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA 540
 Ala Val Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg
 170 175 180

- 39 -

TCC CCG GTG

Ser Pro Val

(2) INFORMATION FOR SEQ ID NO:2:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

Arg Lys Lys Lys Arg Arg

15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 567 base pairs

20 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(ix) FEATURE:

(A) NAME/KEY:

GCG CCC ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG 45

30 Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly

1

5

10

15

TGT ATA ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG 90

Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu

35

20

25

30

- 40 -

GGT GAG GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA 135
 Gly Glu Val Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala
 35 40 45

5 ACG TGC ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA 180
 Thr Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly
 50 55 60

ACG AGG ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT 225
 10 Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr
 65 70 75

ACC AAT GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT 270
 Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly
 15 80 85 90

TCC CGC TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC 315
 Ser Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr
 95 100 105

20 CTG GTT ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT 360
 Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly
 110 115 120

25 GAT AGC AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA 405
 Asp Ser Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu
 125 130 135

AAA GGC TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC 450
 30 Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala
 140 145 150

GTG GGC CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG 495
 Val Gly Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys
 35 155 160 165

- 41 -

GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA 540
 Ala Val Asp Phe Ile Pro Val Glu Asn L u Glu Thr Thr Met Arg
 170 175 180

5 TCC CCG GTG AGA AAG AAG AAG AGA AGA
 Ser Pro Val Arg Lys Lys Lys Arg Arg

(2) INFORMATION FOR SEQ ID NO:4:

10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 603 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

20 (A) NAME/KEY: pBJ1022(His/NS3 (182)/H.T.

ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC ACG GAT CCG CCC ATC 45
 Met Arg Gly Ser His His His His His His Thr Asp Pro Pro Ile
 25 1 5 10 15

ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA ATC 90
 Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile
 20 25 30

30

ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG GTC 135
 Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu Val
 35 40 45

35 CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC ATC 180
 Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala Thr Cys Ile
 50 55 60

- 42 -

AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG ACC 225
 Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg Thr
 65 70 75

5
 ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT GTG 270
 Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn Val
 80 85 90

10
 GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC TCA 315
 Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser
 95 100 105

15
 TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT ACG 360
 Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr
 110 115 120

AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC AGG 405
20 Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg
 125 130 135

GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC TCC 450
25 Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser
 140 145 150

TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC CTA 495
30 Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly Leu
 155 160 165

TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG GAC 540
35 Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val Asp
 170 175 180

- 43 -

TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG GTG 585
Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Val
185 190 195

5 AGA AAG AAG AAG AGA AGA
Arg Lys Lys Lys Arg Arg

10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 630 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(ix) FEATURE:

(A) NAME/KEY: pT5His/HIV/183 No solubilizing motif

ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC GGA TCC CAT AAG GCA 45
25 Met Arg Gly Ser His His His His His His Gly Ser His Lys Ala
1 5 10 15

AGA GTT TTG GCT GAA GCA ATG AGC CAT GGT ACC ATG GCG CCC ATC 90
Arg Val Leu Ala Glu Ala Met Ser His Gly Thr Met Ala Pro Ile
30 20 25 30

ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA ATC 135
Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile
35 40 45

35

ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG GTC 180
Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu Val
50 55 60

- 44 -

CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC ATC 225
 Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala Thr Cys Ile
 65 70 75
 5
 AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG ACC 270
 Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg Thr
 80 85 90
 10
 ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT GTG 315
 Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn Val
 95 100 105
 15
 GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC TCA 360
 Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser
 110 115 120
 20
 TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT ACG 405
 Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr
 125 130 135
 25
 AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC AGG 450
 Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg
 140 145 150
 30
 GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC TCC 495
 Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser
 155 160 165
 35
 TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC CTA 540
 Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly Leu
 170 175 180
 35
 TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG GAC 585
 Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val Asp
 185 190 195

- 45 -

TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG GTG 630
 Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Val
 200 205 210

5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 162 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

15

(ix) FEATURE:

(A) NAME/KEY: NS4A

AGC ACC TGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG 45
 Ser Thr Trp Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala
 1 5 10 15

20

TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC 90
 Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
 25 20 25 30

25

TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC 135
 Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
 35 40 45

30

CAG GAG TTC GAT GAG ATG GAA GAG TGC 162
 Gln Glu Phe Asp Glu Met Glu Glu Cys
 50

35 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 702 base pairs

- 46 -

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: NS3 +NS4A

10 GCG CCC ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG 45
 Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly
 1 5 10 15

TGT ATA ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG 90
 15 Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu
 20 25 30

GGT GAG GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA 135
 Gly Glu Val Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala
 20 35 40 45

ACG TGC ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA 180
 Thr Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly
 50 55 60

25 ACG AGG ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT 225
 Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr
 65 70 75

30 ACC AAT GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT 270
 Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly
 80 85 90

TCC CGC TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC 315
 35 Ser Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr
 95 100 105

- 47 -

CTG GTT ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT 360
 Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly
 110 115 120

5 GAT AGC AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA 405
 Asp Ser Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu
 125 130 135

AAA GGC TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC 450
 10 Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala
 140 145 150

GTG GGC CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG 495
 Val Gly Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys
 15 155 160 165

GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA 540
 Ala Val Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg
 170 175 180

20 TCC CCG GGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG 585
 Ser Pro Gly Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala
 185 190 195

25 TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC 630
 Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
 200 205 210

TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC 675
 30 Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
 215 220 225

CAG GAG TTC GAT GAG ATG GAA GAG TGC 702
 Gln Glu Phe Asp Glu Met Glu Glu Cys
 35 230

(2) INFORMATION FOR SEQ ID NO:8:

- 49 -

	ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT GTG	315
	Ile Ala Ser Pro Lys Gly Pro Val Ile Gln M t Tyr Thr Asn Val	
	95 100 105	
5	GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC TCA	360
	Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser	
	110 115 120	
	TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT ACG	405
10	Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr	
	125 130 135	
	AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC AGG	450
	Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg	
15	140 145 150	
	GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC TCC	495
	Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser	
	155 160 165	
20		
	TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC CTA	540
	Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly Leu	
	170 175 180	
25		
	TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG GAC	585
	Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val Asp	
	185 190 195	
30		
	TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG GGG	630
	Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Gly	
	200 205 210	
	GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG TAT TGC CTG	720
35	Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys Leu	
	215 220 225	

- 50 -

TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC TTG TCC GGG 765
 Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly
 230 235 240

5 AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAG TTC 810
 Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe
 245 250 255

GAT GAG ATG GAA GAG TGC CGG AAG AAA AAG AGA CGC AAG CTT AAT 855
 10 Asp Glu Met Glu Glu Cys Arg Lys Lys Lys Arg Arg Lys Leu Asn
 260

15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 711 base pairs
 20 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(ix) FEATURE:

(A) NAME/KEY:

GCG CCC ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG 45
 Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly
 30 1 5 10 15

TGT ATA ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG 90
 Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu
 20 25 30

35

GGT GAG GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA 135
 Gly Glu Val Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala
 35 40 45

- 51 -

ACG TGC ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA 180
 Thr Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly
 50 55 60
 5

ACG AGG ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT 225
 Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr
 65 70 75

10 ACC AAT GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT 270
 Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly
 80 85 90

TCC CGC TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC 315
 15 Ser Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr
 95 100 105

CTG GTT ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT 360
 Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly
 20 110 115 120

GAT AGC AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA 405
 Asp Ser Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu
 125 130 135

25 AAA GGC TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC 450
 Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala
 140 145 150

30 GTG GGC CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG 495
 Val Gly Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys
 155 160 165

GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA 540
 35 Ala Val Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg
 170 175 180

- 52 -

TCC CCG GGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG 585
 Ser Pro Gly Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala
 185 190 195

5

TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC 630
 Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
 200 205 210

10 TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC 675
 Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
 215 220 225

CAG GAG TTC GAT GAG ATG GAA GAG AAG GAG ACA GAG
 15 Gln Glu Phe Asp Glu Met Glu Glu Lys Glu Thr Glu
 230

(2) INFORMATION FOR SEQ ID NO:10:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 855 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY:

30

ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC ACG GAT CCG GCG CCC
 Met Arg Gly Ser His His His His His His Thr Asp Pro Ala Pro
 1 5 10 15

35 ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA 45
 Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile
 20 25 30

- 53 -

ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG 90
 Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu
 35 40 45

5
 GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC 135
 Val Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala Thr Cys
 50 55 60

10
 ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG 180
 Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg
 65 70 75

15
 ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT 225
 Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn
 80 85 90

20
 GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC 270
 Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg
 95 100 105

25
 TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT 315
 Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val
 110 115 120

30
 ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC 360
 Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser
 125 130 135

35
 AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC 405
 Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly
 140 145 150

TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC 450
 Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly
 155 160 165

- 54 -

CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG . 495
 Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val
 170 175 180

5

GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG 540
 Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro
 185 190 195

10

GGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG TAT TGC 585
 Gly Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys
 200 205 210

15

CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC TTG TCC 630
 Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser
 215 220 225

20

GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAG 675
 Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu
 230 235 240

25

TTC GAT GAG ATG GAA GAG AAG GAG ACA GAG 705
 Phe Asp Glu Met Glu Glu Lys Glu Thr Glu
 245 250

30 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

35 (C) STRANDEDNESS: double

(D) TOPOLOGY: double

(ii) MOLECULE TYPE: cDNA

- 55 -

GA TCA CCG GTC TAG ATCT
T GGC CAG ATC TAGA

5 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
10 (C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (ix) FEATURE:

(A) NAME/KEY:

CCG GTC CCG AAG AAA AAG AGA CGC TAG C
AG GCC TTC TTT TTC TCT GCG ATC G

20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 79 base pairs
25 (B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(ix) FEATURE:

(A) NAME/KEY:

CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAA TTC
GT TAA TAT GGA CTG TCC CTC CAA GAG ATG GTC CTT AAG

35

GAT GAG ATG GAA GAG TGC CGG AAG AAA AAG AGA CGC A
CTA CTC TAC CTT CTC ACG GCC TTC TTT TTC TCT GCG TTC GA

- 56 -

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

10

(ix) FEATURE:

- (A) NAME/KEY: NS4A Active Mutant

Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly Lys

15

5

10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

- (A) NAME/KEY: NS4A Active Mutant

30 Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly Lys

5

10

(2) INFORMATION FOR SEQ ID NO:16:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

- 57 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

5 (ix) FEATURE:

(A) NAME/KEY: Soluble 5A/5B Substrate

Asp Thr Glu Asp Val Val Cys Cys Ser Met Ser Tyr Thr Trp Thr
5 10 15

10 Gly Lys

(2) INFORMATION FOR SEQ ID NO:17:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

25 (A) NAME/KEY: Mutant 5A/5B Substrate

Asp Thr Glu Asp Val Val Ala Cys Ser Met Ser Tyr Thr Trp Thr
5 10 15

Gly

30 (2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

35 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

- 58 -

(ix) FEATURE:

(A) NAME/KEY: Mutant Soluble 5A/5B Substrate

5 Asp Thr Glu Asp Val Val Ala Cys Ser Met Ser Tyr Thr Trp Thr
5 10 15
Gly Lys

2) INFORMATION FOR SEQ ID NO:19:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

15

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

20

(A) NAME/KEY: Soluble 5A/5B Substrate

Asp Thr Glu Asp Val Val Cys Cys Ser Met Ser Tyr Thr Trp Thr
5 10 15
Gly Lys Tyr

25

2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

30

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

35

(ix) FEATURE:

(A) NAME/KEY: Soluble 5A/5B Substrate

- 59 -

Asp Thr Glu Asp Val Val Ala Cys Ser Met Ser Tyr Thr Trp Thr
5 10 15

Gly Lys Tyr

5

2) INFORMATION FOR SEQ ID NO:21:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

(A) NAME/KEY: Soluble 4B/5A Substrate

20

Trp Ile Ser Ser Glu Cys Thr Thr Pro Cys Ser Gly Ser Trp Leu
5 10 15

Arg Asp Ile Trp Asp

25

2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

30 (B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

35

(ix) FEATURE:

(A) NAME/KEY: histidine tag

- 60 -

Met Arg Gly Ser His His His His His Thr Asp Pro

5

10

5 2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

15 (ix) FEATURE:

(A) NAME/KEY: hydrophilic tail

Arg Lys Lys Lys Arg Arg Lys Leu Asn

20

5

2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 4 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

(A) NAME/KEY: hydrophilic tail

35 Lys Glu Thr Glu

2) INFORMATION FOR SEQ ID NO:25:

- 61 -

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

5 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

10 (A) NAME/KEY: hydrophilic tail

Trp Ile Ser Ser Glu Cys Thr Thr Pro Cys Ser Gly Ser Trp Leu

5

10

15

15

Arg Asp Ile Trp Asp

20

(2) INFORMATION FOR SEQ ID NO:26:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 162 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

30 (A) NAME/KEY: NS4A Mutant

GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG TAT TGC CTG 45

Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys Leu

1

5

10

15

35

TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC TTG TCC GGG 90

Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly

20

25

30

- 62 -

AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAG TTC 135
 Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe
 35 40 45

5

GAT GAG ATG GAA GAG TGC
 10 Asp Glu Met Glu Glu Cys
 50

(2) INFORMATION FOR SEQ ID NO:27:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 810 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: pNB182Δ4AHT

25

ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC ACG GAT CCG CCC ATC 45
 Met Arg Gly Ser His His His His His His Thr Asp Pro Pro Ile
 1 5 10 15

30

ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA ATC 90
 Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile
 20 25 30

35 ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG GTC 135
 Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu Val
 35 40 45

- 63 -

CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC ATC 180
 Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala Thr Cys Ile
 50 55 60

5

AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG ACC 225
 Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg Thr
 10 65 70 75

ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT GTG 270
 Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn Val
 80 85 90

15

GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC TCA 315
 Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser
 95 100 105

20

TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT ACG 360
 Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr
 110 115 120

25

AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC AGG 405
 Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg
 125 130 135

30

GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC TCC 450
 Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser
 140 145 150

35

- 64 -

	TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC CTA	495
	Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly Leu	
	155 160 165	
5	TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG GAC	540
	Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val Asp	
	170 175 180	
10	TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG GGG	585
	Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Gly	
	185 190 195	
15	GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG TAT TGC CTG	630
	Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys Leu	
	200 205 210	
20	TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC TTG TCC GGG	720
	Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly	
	215 220 225	
	AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAG TTC	765
25	Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe	
	230 235 240	
	GAT GAG ATG GAA GAG TGC CGG AAG AAA AAG AGA CGC AAG CTT AAT	810
	Asp Glu Met Glu Glu Cys Arg Lys Lys Lys Arg Arg Lys Leu Asn	
30	245 250 255	

(2) INFORMATION FOR SEQ ID NO:28:

- 35 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 162 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

- 65 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5 (ix) FEATURE:

(A) NAME/KEY: Native NS4A

10 TCA ACA TGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG 45
Ser Thr Trp Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala
1 5 10 15

TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC 90
15 Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
20 25 30

TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC 135
Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
20 35 40 45

CAG GAG TTC GAT GAG ATG GAA GAG TGC
Gln Glu Phe Asp Glu Met Glu Glu Cys
50

25

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 33 amino acid residues
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

(A) NAME/KEY: Carboxl 33 mer of NS4A

Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly Lys Pro Ala
5 10 15

5 Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe Asp Glu Met
20 25 30

Glu Glu Cys

10

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 amino acid residues

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

20

(ix) FEATURE:

(A) NAME/KEY: Carboxl 33 mer of NS4A of HCV-BK strain

Ser Val Val Ile Val Gly Arg Ile Ile Leu Ser Gly Arg Pro Ala
25 5 10 15

Ile Val Pro Asp Arg Glu Leu Leu Tyr Gln Glu Phe Asp Glu Met
20 25 30

30 Glu Glu Cys

2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- 67 -

(ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

(A) NAME/KEY: Native 5A/5B Substrate

5

Asp Thr Glu Asp Val Val Cys Cys Ser Met Ser Tyr Thr Trp Thr

5

10

15

Gly

- 68 -

WE CLAIM:

1. A soluble HCV substrate which encodes a nonstructural polyprotein of the HCV genome.
- 5 2. The HCV substrate of claim 1 further comprising a solubilizing motif attached to said substrate
3. The HCV substrate of claim 2 wherein the solubilizing motif is
- 10 comprised of an ionizable amino acid.
4. The HCV substrate of claim 3 wherein the ionizable substrate is either arginine or lysine.
- 15 5. The HCV substrate of claim 4 having a sequence defined by defined by SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, and SEQ ID NO: 21.
6. A nucleic acid encoding the soluble HCV substrate of claims 1, 2, 3, 4
- 20 or 5.
7. A vector containing a nucleic acid which encodes a soluble HCV substrate of claims 1, 2, 3, 4, or 5.
- 25 8. A cell transfected or transformed with a vector containing a nucleic acid which encodes a soluble HCV substrate of claims 1, 2, 3, 4, or 5.

30

35

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(57) Abstract

Soluble HCV nonstructural substrates of the HCV polyprotein.

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GA	Gabon			VN	Viet Nam

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/06389

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/18 C12N9/50 C12Q1/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE,A,42 40 980 (BOEHRINGER MANNHEIM GMBH) 10 February 1994 see page 2, line 46 ---	1
X	WO,A,93 25575 (UNITED STATES OF AMERICA) 23 December 1993 see figure 1A ---	1-4
X,P	WO,A,95 22985 (ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P. ANGELETTI SPA) 31 August 1995 see page 11, line 14 - line 19 --- -/-	1

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

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- *E* earlier document but published on or after the international filing date
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Date of the actual completion of the international search

13 November 1996

Date of mailing of the international search report

22. 11. 96

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Authorized officer

Skelly, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/06389

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X,P	ANTIVIRAL RESEARCH, vol. 32, 1996, pages 9-18, XP000608038 K. SUDO ET AL.: "Establishment of an in vitro assay system for screening hepatitis C protease inhibitors using high performance liquid chromatography" see page 11, column 2, paragraph 2 ---	1
A	WO,A,91 15575 (CHIRON CORPORATION) 17 October 1991 see the whole document ---	1
A	J. VIROL., vol. 68, no. 12, 1994, pages 8147-8157, XP002018452 C. LIN ET AL.: "Hepatitis C virus NS3 serine proteinase" -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PL 1/US 96/06389

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		US-A- 5371017	06-12-94

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